Environmental Science and Pollution Research

Extracellular polymeric substances with metal adsorption capacity produced by Pseudoalteromonas sp. MER144 from Antarctic seawater

--Manuscript Draft--

Manuscript Number:	ESPR-D-17-04631
Full Title:	Extracellular polymeric substances with metal adsorption capacity produced by Pseudoalteromonas sp. MER144 from Antarctic seawater
Article Type:	Research Article
Keywords:	Antarctica; exopolymers; biosorption; Heavy metals; cryoprotection
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Funding Information:	PNRA — Not applicable
	MNA — Not applicable
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Extracellular polymeric substances with metal adsorption capacity produced by *Pseudoalteromonas* sp. MER144 from Antarctic seawater

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For submission to *Environmental Science and Pollution Research*

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Abstract

The EPS-producing *Pseudoalteromonas* sp. MER144 was selected among 606 isolates from Antarctic seawater for the highly mucous appearance of its colonies on agar plates. The production of EPSs was enhanced by a step-by step approach varying the carbon source, substrate and NaCl concentrations, temperature and pH. Optimal conditions for the EPS production resulted at 4 °C and pH 7 in the presence of sucrose (2 %, w/v) and NaCl (3 %, w/v). EPSs produced under optimal conditions were chemically characterized, resulting in a moderate carbohydrate content (35 %), uronic acids (14 %) and proteins (12 %). Monosaccharide composition was estimated to be Glu:Man:GluN:Ara:GluA:GalA:Gal (1:0.36:0.26:0.06:0.06:0.05:0.03), while the estimated molecular weight was about 250 kDa. The addition of sucrose in the culture medium, by stimulating the EPS production, allowed MER144 to tolerate higher concentrations of mercury and cadmium. This finding was probably dependent on the presence of uronic acids and sulfate groups, which can act as ligands for cations, in the extracted EPSs. Monitoring EPS production under optimal conditions at different concentrations of mercury and cadmium revealed that EPS amounts increased at increasing heavy metal concentrations, indicating an adaptation to the stress conditions tested.

Keywords: Antarctica, exopolymers, heavy metals, cryoprotection

Introduction

The occurrence of heavy metals has been reported in ice cores and snow pack in Antarctica (Marteel et al. 2008; Hur et al. 2007). It is mainly due to natural sources as such as gaseous emissions from the oceans, bacterial methylation of metals with production of highly volatile compounds (Pongratz and Heumann 1999), submarine volcanoes activities, wet deposition of windborne soil particles and direct release from the sea ice (Grotti et al. 2005). These natural background levels are now being disturbed by anthropogenic influences. The Antarctic continent, which has been viewed for a long time as a pristine and isolated environment, is unfortunately experiencing increasing contaminant influxes that are likely to become more severe in the future. Heavy metals have been mainly detected in the 2 % of ice-free lands of the continent, where most of the human activities occur, in the waters of the Southern Ocean bordering Antarctica, in the sedimentary compartment, also in the Terra Nova Bay area (Fuoco et al. 1994, 1995; Giordano et al. 1999; Dalla Riva et al. 2004), in addition to the concentrations accumulated in the biota (Capon et al. 1993; Bargagli et al. 1996, 1998; de Moreno et al. 1997; Negri et al. 2006).

Microorganisms have evolved various responses to the heavy-metal stress (Huang and Liu 2013; Pintor et al. 2012), with the secretion of extracellular polymeric substances (EPSs) that are among more efficient ones (Wei et al. 2011). EPSs are involved in various biological functions, as such as cell adhesion to substrata and protection from predation, extreme temperatures and antibiotics. Moreover, EPSs serve as biosorbing agents because they accumulate nutrients from the surrounding environment and play a crucial role in biosorption of heavy metals. Due to their polyanionic nature, EPSs form complexes with metal cations resulting in the metal immobilization within the exopolymeric matrix. Such complexes generally result from electrostatic interactions between the metal ligands and negatively charged components of biopolymers (Pal and Paul 2008). Then, detoxification of heavy

metals can occur by their transformation by enzymatic activities and subsequent precipitation in the polymeric mass. This wide spectrum of functional activities is reflected not merely in the complex chemistry of these biopolymers, but also in the diversity of bacterial genera that are able to produce them (Rosenberg and Ron 1999). In recent years, members within the genus *Pseudoalteromonas* have been reported as producers of exopolymers with different chemical and physical characteristics, and with important biotechnological properties (Nazarenko et al. 2003; Mancuso Nichols et al. 2004; Mancuso Nichols et al. 2005c). However, poor are the reports that describe deeply their properties, and the information on the metal-binding action of exopolymers produced by *Pseudoalteromonas* species are also very limited (Loaëc et al. 1998).

In this study, the EPS production by a *Pseudoalteromonas* isolate (strain MER144) from Antarctic seawater was analyzed in relation to heavy metal tolerance. The EPS potential as bacterial cell cryoprotectants was also explored.

Material and Methods

Bacterial strain

The EPS-producing *Pseudoalteromonas* sp. MER144 (Accession Number HQ534322; Lo Giudice et al. 2012) was selected for further characterization among 606 isolates from Antarctic seawater (Terra Nova Bay, Ross Sea; 15m depth) as it appeared highly mucous on Marine Agar (MA; Difco) plates and in Marine Broth (MB; Difco) liquid cultures supplemented with sugars, as described below. *Pseudoalteromonas* sp. MER144 belongs to the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA) kept at the University of Messina (Italy).

EPS production by *Pseudoalteromonas* sp. MER144

EPS production by *Pseudoalteromonas* sp. MER144 was assayed in the absence and presence of sugars (i.e. glucose, mannose or sucrose; 0.6 %, w/v) on MA plates and in MB liquid cultures. Results were recorded after incubation at 15 °C for 14 days (Fusconi and Godinho 2002; Loaëc et al. 1998). Sugars were sterilized separately by autoclaving at 121 °C for 15 min, and added to the sterile media at 50 °C at the desiderated final concentration. Plates were observed under stereoscopic microscope to detect the possible mucous aspect of colonies (Yildiz et al. 2014), while liquid cultures were checked for viscosity to the naked eye. A supplementary test was performed to highlight the presence of a slime related to the EPS production, according to the method by Christensen et al. (1985). Two mL of MB in borosilicate test tubes were inoculated with a loopful of microorganisms from agar plates and incubated at 15 °C for 14 days. Culture were then decanted, washed with distilled water (pH 7.3) and left to dry at room temperature. Afterward, the tubes were stained with a safranine solution (4 %, v/v). Each tube was then gently rotated to ensure the uniform staining and then the contents were gently decanted. The tubes were placed upside-down to drain and then observed for biofilm formation, which was considered positive when a visible film lined the wall and the bottom of the tubes. Ring formation at the liquid interface was not considered as indicative of biofilm formation.

Enhancement of EPS production

To individuate the optimal growth conditions (in terms of carbon source, temperature, NaCl concentration and pH) for the EPS production, a step-by-step approach was used. At each step the optimal value recorded for the previously tested parameter was retained. For each test, a bacterial pre-culture (10 %, v/v) in the exponential phase was used to inoculate 300 mL of a minimal medium, which contained (per liter of Väätänen nine-salt solution, VNSS): 0.5 g peptone, 0.1 g yeast extract and a carbon source (the carbon source and its concentration were

selected on the basis of experimental needs, as specified below) (Holmström et al. 1998). The VNNS solution contained (per liter of distilled water): 17.6 g NaCl, 1.47 g Na_2SO_4, 0.08 g $NaHCO_3$, 0.25 g KCl, 0.04 g KBr, 1.87 g $MgCl_2$ x $6H_2O$, 0.41 g $CaCl_2$ x $2H_2O$, 0.008 g SrCl $6H_2O$, 0.008 g H_3BO_3 (pH 7). Cultures were incubated at 4 and/or 15 °C, as specified below for each step, for one month. At regular intervals, 9 mL were sampled from the culture broth to evaluate: 1) bacterial growth by UV-visible optical density measurements (UV-mini-1240, Shimadzu at 600 nm (OD600) and 2) EPS-production by applying the phenol-sulphuric acid method on cell free broth, with use of glucose as a standard (Dubois et al. 1956).

The effect on the EPS production of three different carbon sources (i.e., glucose, mannose or sucrose; 0.6 %, w/v) was first evaluated at 15 °C. By growing the strain in the presence of the preferred carbon source at the optimal concentration for EPS production, the influence of the other variables was investigated in the following order: concentration of the carbon source (i.e., 0.6, 1 and 2 %, w/v; maintaining the incubation temperature at 15 °C), temperature (4 and 15 °C), pH (6, 7 and 8) and NaCl concentration (1, 3 and 5 %, w/v).

EPS extraction from the culture medium

For the extraction of the EPSs from the bacterial cultures *Pseudoalteromonas* sp. MER144 was grown under the optimal conditions determined by the step-by step approach. Cells were harvested from 1L cultures in the stationary phase of growth by centrifugation (8,000xg for 10 min at 4 °C). The liquid phase was treated with 1 volume of cold ethanol added drop by drop under stirring. Alcoholic solution was kept at -20 °C overnight and then EPS was obtained by centrifugation at 10,000 x g for 30 min. The pellet was dissolved in hot water. The same procedure was repeated again. The final water solution was dialyzed against tap water (48 h) and distilled water (24 h), then freeze-dried and weighted.

EPS characterization

Colorimetric assays

Extracted EPSs were assayed for total carbohydrate (CHO), protein (PRT) and uronic acid (UA) content. CHO content was detected by the Dubois method (Dubois et al. 1956), and expressed in D(+)-glucose equivalents after reaction with 96 % sulphuric acid and 5 % phenol, followed by a spectrophotometric detection at λ490 nm. PRT content was spectrophotometrically determined using Coomassie Brilliant Blue (Bradford 1976). After reaction with the dye, absorbance was determined at λ595nm. PRT concentrations are reported in bovine serum albumin (BSA, Biorad) equivalents. Finally, UA amount was detected using glucuronic acid as a standard by using and spectrophotometric detection at λ525nm (Filisetti-Cozzi and Carpita 1991).

Monosaccharide analysis

For the sugar analysis, lyophilized samples (3–4 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. Sugar components were identified by thin-layer chromatography and high-pressure anion-exchange pulsed amperometric detection (HPAE-PAD) with sugar standards for identification and calibration curves (Finore et al. 2016).

Nuclear Magnetic Resonance (NMR) spectroscopy

1H- and 13C-NMR spectra of the purified EPS samples (10 mg mL^{-1} D2O) were recorded on a Bruker AMX-600 MHz at 50 °C (Mastascusa et al. 2014). Briefly, the samples were exchanged twice with D_2O with an intermediate lyophilization step and finally dissolved in 500 μL of D_2O. Chemical shifts were reported in parts per million (ppm) with reference to D_2O and to CH_3OH, for 1H and 13C spectra, respectively.

FT-IR spectroscopy

The purified EPS samples were characterized by the Fourier transform-infrared (FT-IR) spectra analysis. Spectra were collected with a resolution of 4 cm^{-1} in the 4000-400 cm^{-1} region, on pellets obtained from a mixture of the polysaccharides (2 mg) and dried KBr (200 mg), subsequently pressed into a 16 mm diameter mold (Mancuso Nichols et al. 2004). Sulfate content was determined according to the method of Lijour et al. (1994), by relating the absorbance of band at 1250 cm^{-1} (attributed to sulphate stretching vibrations) and that of the band 1050 cm^{-1} (due to complex vibration modes of polysaccharides). The relation applied was: Abs (1250) / Abs (1050) = % sulfate x 0.027 (±0.004) + 0.36 (±0.06).

EPS molecular weight

EPS molecular weight was estimated by gel filtration on Sepharose CL-6B column (lx80 cm) using H2O/Pyridine/Acetic acid (500/5/2, by vol) as eluant, with a flux of 3.7 mL h^{-1}, and density gradient centrifugation method, using a sucrose gradient from 0 to 50 % w/v at 13,000 g for 16 h (Yildiz et al. 2014). In both methods 10 mg of EPS and a mixture of dextran for calibration curves (10 mg of T-700, MW 670,000; T-400, MW 410,000; T-150, MW 154,000) were used.

Biotechnological potential of EPSs

EPSs as heavy metal chelating agents

Tolerance to four heavy metals (HMs; ie., cadmium, mercury, zinc and iron; range 10-10,000 ppm) was tested by the plate diffusion method (Selvin et al. 2009) by comparing bacterial growth on a medium that contained (0.6 %, w/v; SUC +) or did not contain (SUC –) sucrose (which resulted the preferred sugar for the EPS production). Briefly, 0.5 mL of appropriate metal salt solution (in sterile phosphate buffer saline; PBS) was added in a central well of 1

cm in diameter and 4 mm in depth. The bottom of each well was sealed with soft agar (0.8 % agar, w/v). Sterile PBS was used as a negative control. Plates were then pre-incubated at 37 °C for 24 h to allow diffusion of the metal into the agar and the formation of a concentration gradient in the media around the well. *Pseudoalteromonas* sp. MER144 was inoculated in radial streaks in duplicate. Plates were then incubated at 4 °C for 21 days. After incubation, the area of growth inhibition (in mm) was measured as the distance from the edge of the central well to the leading edge of the growing colonies. The percentage of bacterial resistance was calculated in terms of the ratio: length of the growth in mm *vs* length of the total inoculated streak. Tolerance range were classified in complete (100 % of growth), high (≥50-99 % of growth), low (≥1-49 % of growth) or absent (no growth; 0 %) (Mangano et al. 2014).

Heavy metal influence on EPS production

Based on preliminary results obtained on HM tolerance, the effect of the initial concentration of Cd and Hg (less tolerated metals) on the EPS production was evaluated by growing *Pseudoalteromonas* sp. MER144 under the optimal growth conditions, as previously determined. Bacterial growth and EPS production were quantitatively monitored in 300 mL culture as described above, and the effect of heavy metals was detected by using the same metals and concentrations used for the tolerance test.

Additionally, the chelating activity of EPSs towards cadmium was evaluated by dissolving 50 mg of extracted EPSs in 40 mL of MilliQ water, and then mixing with a cadmium solution (500 ppm, w/v) (Loaëc et al. 1997). The solution was shacked at 200 rpm by using a rotary shaker for 3 h, until an equilibrium sorption was reached. Residual metal was detected in 1 mL of solution filtered on membrane filters (Millex syringe filters, pore size 0.45 μm, Millipore), then acidified with 1 % nitric acid solution. The final metal concentration (Meeq)

was determined by using a mass spectrophotometer ICP-MS (Fisons Instruments), leading to the calculated values for biosorbent metal uptake in sorption system using the general equation: (Mei – Meeq)/m x V, where Mei is the initial metal concentration in solution of volume V, and m is the mass of EPSs. Appropriate negative controls were treated as described to ensure the absence of glassware sorption of the metals.

EPSs as cryoprotective agents

To test the cryoprotective effect of EPSs, isolates were grown under optimal conditions for EPS production until they reached the exponential phase, according to Li et al. (2006).

In order to obtain bacteria with and without EPSs, culture broths were centrifuged at 10000xg for 20 min at 4 °C. The presence (EPS +) or absence (EPS –) of polysaccharides around the bacteria cell wall was checked under light microscope after staining with Alcian blue and Congo red. Then, biomasses (1 mL) were frozen at -20 °C in sterile tubes and thawed at room temperature. The freeze-thaw cycle was repeated for four consecutive times. At the end of each thawing, bacterial viability in MB inoculated with bacterial biomass was spectrophotometrically tested (OD600). MB inoculated with untreated bacteria was used as a control.

Results

Enhancement of EPS production by *Pseudoalteromonas* sp. MER144

Pseudoalteromonas sp. MER144 produced EPSs when growing in both solid and liquid media, showing an evident mucoid aspect on agar plates and producing a slime in tubes.

The EPS production always appeared to be correlated to the optical density values with the highest EPS amounts that generally occurred during the exponential phase of growth. An evident increase in EPS production was determined by the addition of sucrose in the culture

medium (Fig. 1A). A continuous increase in growth was observed up to 384 h of incubation, while EPS production increased up to 96 h. Then, while a sharp decline in EPS production was observed, the decline in growth was marginal.

The concentration of sucrose also influenced the EPS production. EPS amounts of 105.8 and 214.2 mg L^{-1} were obtained by growing *Pseudoalteromonas* sp. MER144 in the presence of 0.6 and 2 % of sucrose, respectively after 96 and 168 h of incubation. In the presence of mannose and glucose the EPS amounts resulted lower being 42.8 (after 96h of incubation) and 42.0 mg L^{-1}, respectively (after 384 h).

The effect of temperature was evaluated by using sucrose at a final concentration of 2 % (w/v), as previously determined. The growth pattern resulted similar at both incubation temperatures, whereas an evident increase in EPS production from 214.1 to 318.2 mg L^{-1} was observed at 15 and 4 °C, respectively (Fig. 1B).

The pH did not seem to severely affect the ability of *Pseudoalteromonas* sp. MER144 to produce EPSs, even if pH 7 was chosen as optimal for *Pseudoalteromonas* sp. MER144 (Fig. 1C). Conversely, a weak influence on bacterial growth and EPS production was recorded by varying the NaCl concentration in the medium, with an optimum result that was obtained at 3 % NaCl (300.4 mg L^{-1} after 96 h of incubation) (Fig. 1D). Interestingly, the strain produced EPSs also in condition of iposalinity and hypersalinity, despite the lower amounts. The optimal conditions for EPS production by *Pseudoalteromonas* sp. MER144 are summarized in Table 1. Briefly, the strain produced up to 318.62 mg EPS L^{-1} after 96h incubation (exponential phase) when growing at 4 °C and pH 7, in the presence of 2 % (w/v) sucrose and 3 % (w/v) NaCl.

Characterization of produced EPSs

EPS extraction and chemical characterization

Pseudoalteromonas sp. MER144 was grown in batch culture (1 L) under the optimal conditions reported in Table 1. EPS extraction was performed in the phase of maximum production, as spectrophotometrically determined, allowing to a total amount of lyophilized exoproducts of 100 mg L^{-1}. EPS was highly compact, viscous and poorly soluble in water. Carbohydrates, proteins and uronic acids in the purified EPS accounted for 18, 12 and 14 % w/w, respectively, as determined by colorimetric assays. The HPAE-PAD analysis revealed that the EPS was constituted of glucose, mannose, galactosamine, arabinose, glucuronic acid, galacturonic acid and galactose in 1:0.36:0.26:0.06:0.06:0.05:0.03 relative molar ratio. The estimated molecular weight was about 250 KDa. The FT-IR and NMR analysis confirmed the heterogenous nature of the isolated polymer. Indeed, the FT-IR spectrum (Fig. 2) revealed the presence of peaks between 1650 e 1050 cm^{-1}, attributable to the exopolysaccharide besides the signals relative to the presence of amino sugars and proteins (1550 cm^{-1}). Sulfate content was close to 3.1 %. The analysis of the 1H-NMR (Fig. 3A) spectrum of the EPS confirmed the presence of the monosaccharide units evidenced by chemical analysis: indeed inter alia in the anomeric region, six main signals at δ 5.62 ppm, 5.6 ppm, 5.60 ppm, 5.57 ppm, 5.38 ppm and 5.33 ppm were tentatively assigned to α-Glc, α-Man, α-GlcN, α-Ara, α-GlcA, α-GalA and α-Gal residues, respectively. The analysis of the 13C-NMR spectrum (Fig. 3B) showed three signals at 103.02 ppm, 93.07 ppm and 82.27 ppm in the anomeric region, attributable to the presence of the three most abundant monomers i.e. α-Glc, α-Man and α-GlcN.

Biotechnological potential of EPS

EPSs as heavy metal chelating agents

HM tolerance was in the order Hg<Cd<Zn<Cu<Fe, even if it resulted always higher in the medium amended with sucrose (SUC +). *Pseudoalteromonas* sp. MER144 completely (100 % of growth) tolerated Fe up to 10000 ppm, Cu up to 7500 ppm, Cd and Zn up to 2500 ppm, and

Hg up to 500 ppm, respectively (Table 2). In particular, the tolerance to Cd and Hg increased in the presence of the sugar in the medium. For this reason, the influence of different concentrations of Cd and Hg on EPS production was investigated in liquid cultures by growing *Pseudoalteromonas* sp. MER144 under the optimal conditions determined above (Fig. 4). EPS amounts were estimated after a 96 h incubation (exponential phase of growth). Increasing in heavy metal concentrations positively influenced the EPS production by *Pseudoalteromonas* sp. MER144. In the case of the Hg-amended medium, EPS amounts were always higher than the control (i.e. the medium not amended with metals) up to 2500 ppm. At 0 and 50 ppm of Hg EPS amounts increased from 296 to 419 mg L^{-1}. In the presence of Cd, a regular and evident increase in EPS amount was observed between 0 and 1000 ppm, reaching the maximum value of 437 mg L^{-1}.

Finally, the chelating activity of the EPS produced by *Pseudoalteromonas* sp. MER144 on cadmium salts was investigated (Fig. 5). The EPS removed a quantity of cadmium, present in an aqueous solution, equal to 34 % after 5 min. The adsorption slowly continued up to 120 min, even if it reached the highest level of metal removal (48 %) after 60 min. After that, a state of equilibrium was achieved.

EPSs as cryoprotective agents

The study of EPS effects on cell survival ratio showed differences (up to 25 % of OD values) in bacterial growth between EPS – and EPS + cultures already after the first and second freezing/thawing cycles, reaching a value of 50 % after the fourth cycle (Fig. 6).

Discussion

Members in the genus *Pseudoalteromonas* have important ecological implications in the marine environment, playing a role in the control of microbial community as producers of

bioactive molecules endowed with antifouling and antagonistic activities. Thus, representatives of this genus appear to be particularly promising for biotechnological applications. Several *Pseudoalteromonas* strains have been isolated from Antarctica, inshore waters, sediment or surfaces of marine organisms, and were shown to synthesize a wide range of bioactive molecules. To date only few bacterial EPSs from the Antarctic marine environment have been characterized, but most producers just belong to the genus *Pseudoalteromonas* (Corsaro et al. 2004; Mancuso Nichols et al. 2004, 2005a; Kim and Yim, 2007). In this study, several hundred isolates from Antarctic seawater were screened for EPS production and displayed a mucoid morphology on media supplemented with sugars (data not shown). *Pseudoalteromonas* sp. MER144, which showed the best growth and enhanced mucoid morphology in the presence of sugars, was selected for further characterization and the EPS production was then put in relation to heavy metal tolerance.

Pseudoalteromonas sp. MER144 produced the highest amounts of exoproducts during the exponential phase of growth, as previously observed for *Pseudoalteromonas* sp. S-15-13 and *Pseudoalteromonas* antarctica NF3 from Antarctica (Bozal et al. 1994; Li et al. 2006). This could be related to the production of EPSs in a capsular form, assuming protective functions for the cells, by preserving them from predation, heavy metals and acidic pH values in the bulk environment (Mancuso Nichols et al. 2005a).

Culture conditions could strongly influence the EPS biosynthesis in terms of chemical structure, physico-chemical properties, molecular mass and monosaccharide ratio, as well as by a quantitative point of view (Corsaro et al. 2004; Mancuso Nichols et al. 2004, 2005a). Monitoring exoproduct production over time allow us to recover higher EPS amounts at increasing carbon source concentrations and low temperature. In line with results obtained by Caruso et al. (submitted) and Li et al. (2006) for Antarctic marine bacteria, pH and NaCl concentrations only slightly influenced the biosynthetic activity with the higher EPS amounts

that were achieved at pH 7 and NaCl (3 %, w/v). Among assayed parameters, the carbohydrate availability appeared to be an important limiting factor and sucrose resulted the optimal source for the EPS synthesis by *Pseudoalteromonas* sp. MER144. This finding is in line with previous observations on Antarctic sponge-associated bacteria (Caruso et al. submitted) and the marine *Hahella chejuensis* (Ko et al. 2000). As stated above, an increase in the EPS yield was observed by increasing the sugar concentration (from 0.6 to 2 %; w/v), thus confirming that higher C/N ratio could represent an important factor for the EPS production (Kumar et al. 2007). With respect to temperature, despite *Pseudoalteromonas* sp. MER144 grew slower at 4 than at 15 °C, the higher EPS production was enhanced at 4 °C, suggesting a possible cryoprotective role played by the EPSs at suboptimal growth temperature. This finding was supported by data obtained by several authors studying cold-adapted bacteria (Kumar et al. 2007; Qin et al. 2007; Mancuso Nichols et al. 2005b). For examples, Mancuso Nichols et al. (2005b) reported that EPS amounts produced by *Pseudoalteromonas* strains were thirty times higher at suboptimal growth temperatures (i.e. -2 and 10 °C) that at 20 °C. Li et al. (2006) observed that the Antarctic psychrotrophic *Pseudoalteromonas* S15-13 better produced EPSs at 8 °C. Finally, Marx et al. (2009) reported on the increased EPS production by the psychrophilic *Colwellia psychrerythraea* strain 34H from Arctic marine sediment under stressful environmental conditions. In this study, the produced EPSs allowed *Pseudoalteromonas* sp. MER 144 cells to survive to repeated freeze-thawing cycles, thus making these molecules potential cryoprotective agents to be exploited in the medical and food industrial fields. Similar results were obtained by Selbmann et al. (2002) for the Antarctic fungus *Phoma herbarum*, Marx et al. (2009) for *Colwellia psychrerythraea* 34H from Arctic sediment, and Li et al. (2006) for *Pseudoalteromonas* sp. S15-13 from Antarctic ice. In Polar regions cold-adapted microorganisms must cope with frequent freeze-thaw cycles, which tend to damage living cells and attenuate cell viability (Kim and Yim 2007),

and are accustomed to being frozen within their habitats. EPSs can form and maintain a protective microhabitat around microorganisms in cold environments. Krembs et al. (2002), for example, suggested that high concentrations of EPSs in the Arctic sea-ice brine channels may provide buffering against harsh winter conditions and high salinity, and cryoprotect the microbes living there against ice-crystal formation.

In recent years, the characterization of bacterial EPSs from polar environments has become an active research field, but few EPSs from cold-adapted psychrotolerant marine bacteria have been characterized to date (Carriòn et al. 2015). In this study, both the amount and chemical composition of the EPSs extracted from *Pseudoalteromonas* sp. MER144 cultures grown under optimal conditions were in line with those reported in literature (Li et al. 2006; Mata et al. 2006), even if higher carbohydrate and lower protein concentrations have been generally reported for Antarctic bacteria (Mancuso Nichols et al. 2004, 2005). The high protein content suggests a possible application as emulsifying agents (Bouchotroch et al. 2000; Mata et al. 2006). The HPAE-PAD analysis revealed as principal constituents of the EPSs galactosamine, glucose, mannose, and arabinose in different a molar ratios. The presence of glucose residues is a common feature in microbial polysaccharides (Carrion et al. 2015; Kim and Yim 2007; Mancuso Nichols et al. 2004), while mannose and galactosamine have been frequently reported as the main constituent in different EPSs produced by cold-adapted marine bacteria (Mancuso Nichols et al. 2004; Liu et al. 2013). The monosaccharide composition appears to be variably composed from that of the EPSs produced by a number of marine bacteria. Liu et al. (2013) reported on a EPS by the Arctic *Pseudoalteromonas* sp. SM20310 that was mainly composed of mannose, and traces of glucose, galactose, rhamnose, N-acteylglucosamine, N-acetylgalactosamine and xylose. A similar chemical composition was found for EPSs produced by other two *Pseudoalteromonas* isolates consisting of mannose and traces of glucose (Corsaro et al. 2004), and galactose and glucose (Kim and Yim 2007). More complex

structures were evidenced by Mancuso Nichols et al. (2004, 2005a) who reported the presence of sulfate groups, high levels of uronic acids and a different monosaccharide composition for EPSs produced by *Pseudoalteromonas* isolates. Overall, the presence of different sugar moieties suggests that the EPSs were heteropolysaccharides, while the occurrence of non-sugars (e.g. uronic acids, sulfates, and proteins) indicates the acidic nature of the EPSs. In the marine environment these features confer a negative charge and a sticky quality to the EPSs, which may act as ligands for cations such as dissolved metals (Pal and Paul 2008). *Pseudoalteromonas* sp. MER144 resulted multi-tolerant to heavy metals, both in the presence and absence of sucrose. This finding confirms previous results reported by De Souza et al. (2006) and Lo Giudice et al. (2013) for bacteria from Antarctic seawater and sediment, respectively. The heavy metal toxicity of *Pseudoalteromonas* sp. MER144 was in the order Hg>Cd>Zn>Cu>Fe. As it is well known, microorganisms can develop resistance in the growing presence of toxic compounds, including heavy metals, in the environment (Nair et al. 1992). This could explain the higher tolerance level showed by the isolate towards Zn, Cu and Fe, which are essential elements for the microbial life which occur at high concentrations in the Antarctic environment (De Souza et al. 2006). Similarly, the higher toxicity showed by Hg and Cd could derive from their absence or poor concentration in Antarctic matrices (Bargagli et al. 1996). The increased EPS production at increasing Hg and Cd concentrations in the culture media could represent an organism adaptation to the tested stress conditions (Priester et al. 2006), by reducing the concentration of free ions, which are chelated by the EPSs, in the bulk environment and, in turn, their toxicity (Kim et al. 1999). Similar results were obtained by Ozturk and Aslim (2008), who reported a higher EPS production in the presence of higher Cr concentration for *Chroococcus* and *Synechocystis*, Kazy et al. (2002) for a Cu-resistant *Pseudomonas aeruginosa* strain, Kiliç and Dönmez (2008) for Cr-resistant strains affiliated to the genera *Pseudomonas*, *Micrococcus*, and *Ochrobactrum*.

More interestingly, the EPSs produced by *Pseudoalteromonas* sp. MER 144 were able to remove cadmium from an aqueous solution with a removal percentage of 48 %. This percentage is similar to that reported by Noghabi et al. (2007) for *Pseudomonas fluorescens*, but lower than that observed for the marine bacterium *Enterobacter cloaceae* (65 %; Iyer et al. 2005). Despite the mechanism of action and the potential effect on the environment require further investigation, the exopolymers produced by *Pseudoalteromonas* sp. MER 144 could possess an exploitable application in the bioremediation of heavy metals-contaminated marine environments. In conclusion, this work contributes to increase our knowledge on the ecological roles (i.e. cryoprotection and heavy metal sequestration) and biotechnological uses (i.e. as cryoprotective agents and heavy metal chelators) of EPSs from Antarctic bacteria.

Acknowledgments

This research was supported by grants from PNRA (Programma Nazionale di Ricerche in Antartide), Italian Ministry of Education and Research (Research Project PNRA 2004/1.6), and from MNA (Museo Nazionale dell'Antartide).

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Figure captions

Fig. 1. Influence of different parameters on *Pseudoalteromonas* sp. MER144 growth (lines) and EPS production (histograms). **A)** Incubation at 15 °C in the presence of different carbon sources (0.6 % w/v); **B)** incubation at different temperatures in the presence of 2 % (w/v) sucrose; **C)** incubation at different pH values in the presence of 2 % (w/v) sucrose at 4 °C; **D)** incubation at different NaCl concentrations in the presence of 2 % (w/v) sucrose and pH 7 at 4 °C.

Fig. 2. Fourier transform infrared spectroscopic spectrum of extracellular polymeric substances (EPSs) produced by *Pseudoalteromonas* sp. MER144.

Fig. 3. NMR analysis of EPS produced by *Pseudoalteromonas* sp. MER 144. ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra were registered in D_2O at temperature of 50 °C. Chemical shifts are reported in parts per million (ppm) with reference to D_2O and to CH_3OH, for ^{1}H and ^{13}C spectra, respectively.

Fig. 4. Growth of the EPS-producing *Pseudoalteromonas* sp. MER144 after four freezing/thawing cycles. The black bar indicates OD values of MB inoculated with untreated bacteria (unfrozen).

Fig. 5. Effect of Cd and Hg concentrations on EPS production by *Pseudoalteromonas* sp. MER 144 after a 96 h incubation under optimal conditions (4 °C, pH 7, 2 % sucrose and 3 % NaCl).

Fig. 6. Cadmium adsorption activity of EPSs produced by *Pseudoalteromonas* sp. MER 144. Cd starting concentration 500 ppm.

Conflict of interest

The authors declare they have any conflict of interest.

Figure 1

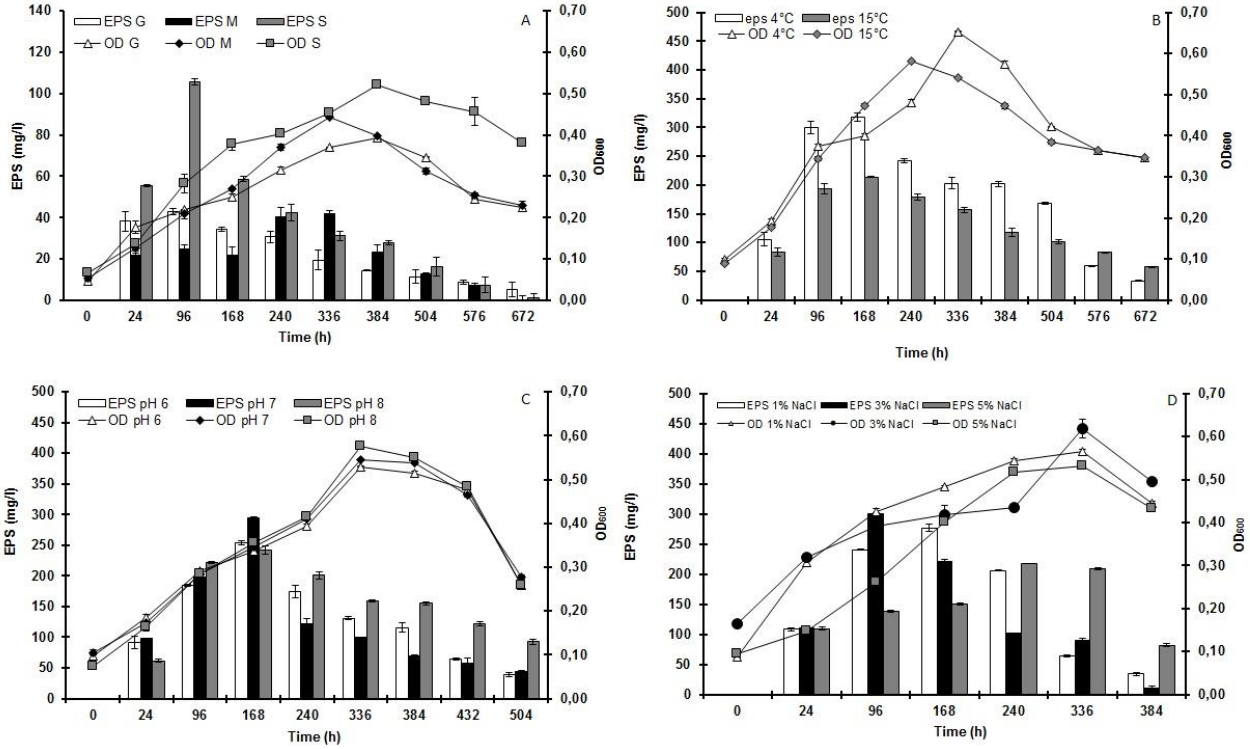

Fig. 1. Caruso et al.

Figure 2

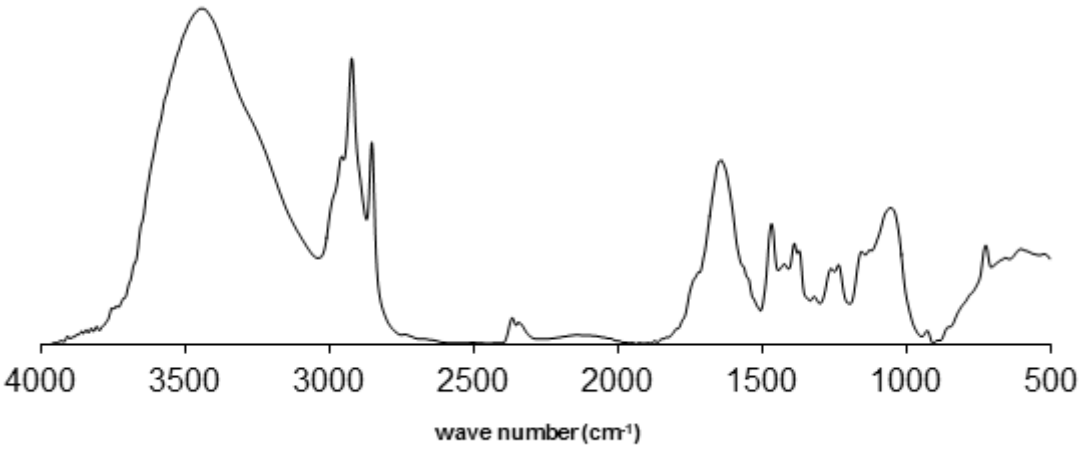

Fig. 2. Caruso et al.

Figure 3

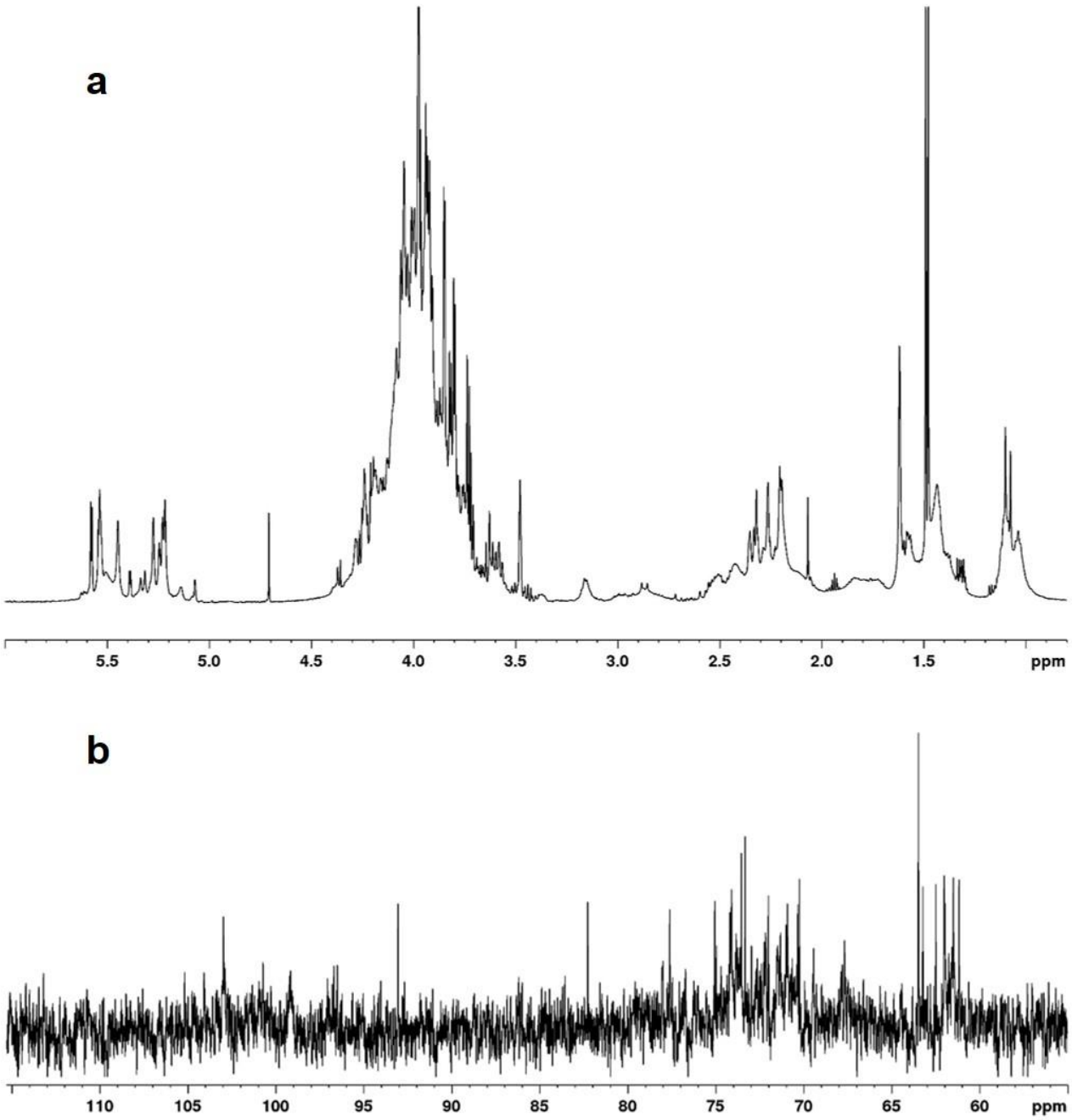

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Figure 4

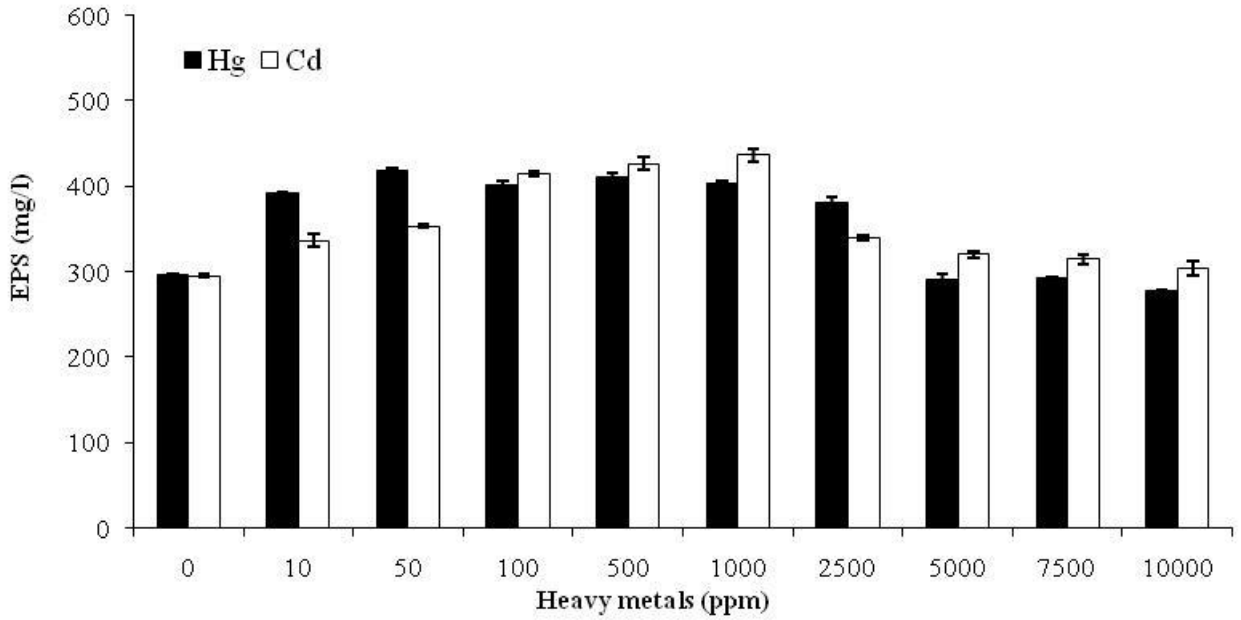

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Figure 5

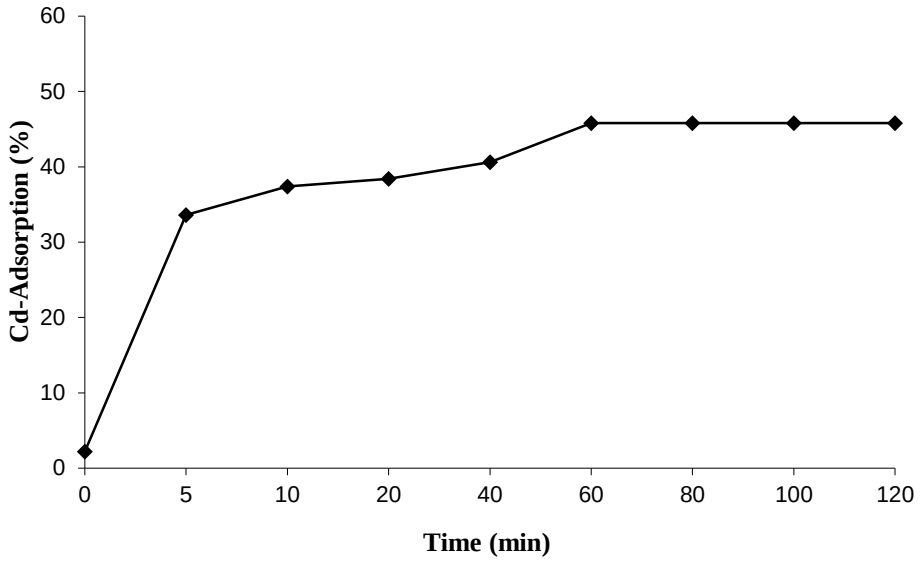


Fig. 5. Caruso et al.

Figure 6

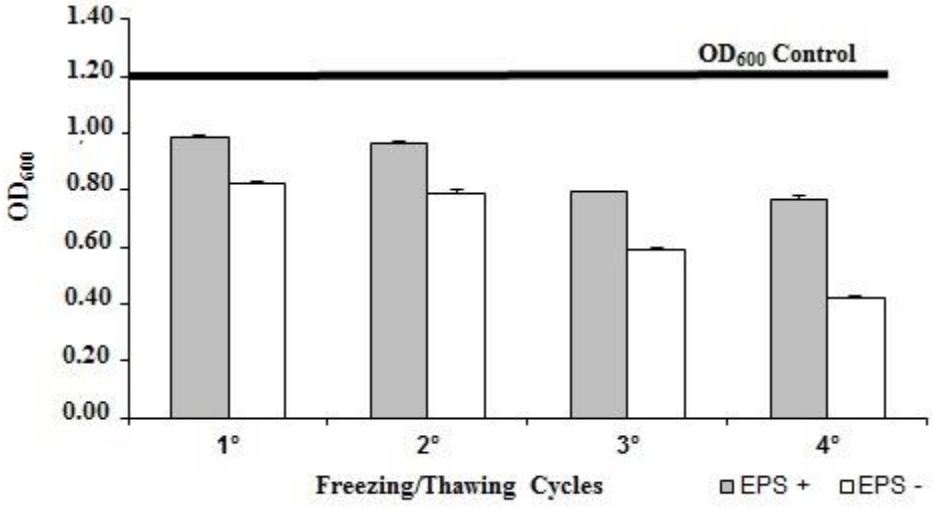


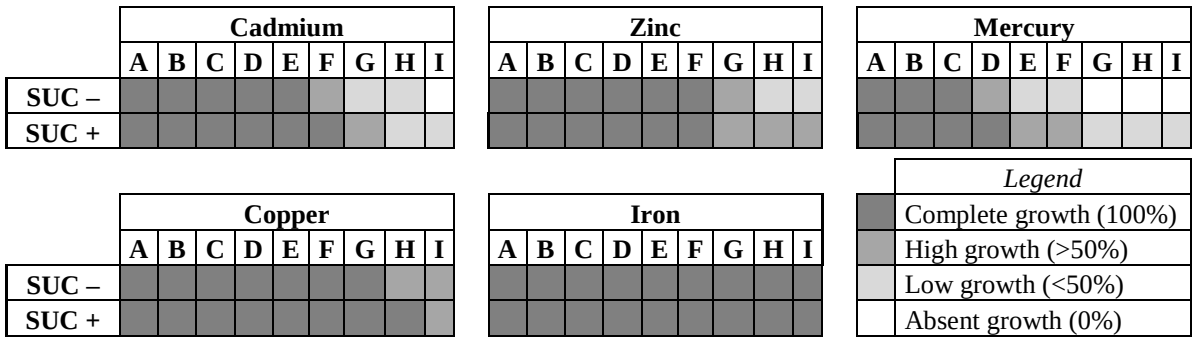
Fig. 6. Caruso et al.

Table 1

Table 1. Optimal conditions for EPS production by *Pseudoalteromonas* sp. MER144 (in bold the optimal conditions that were determined the step-by-step approach).

Variable		conc. (%)	T (°C)	pH	NaCl (%)	EPS (mg/l)
Carbon source	glucose	0.6	15	7	3	42.89
	mannose	0.6	15	7	3	42.04
	sucrose	**0.6**	**15**	**7**	**3**	**105.86**
Carbon source concentration	sucrose	1	15	7	3	76.68
	sucrose	**2**	**15**	**7**	**3**	**214.16**
Incubation temperature	**sucrose**	**2**	**4**	**7**	**3**	**318.26**
	sucrose	2	15	7	3	214.16
pH value	sucrose	2	4	6	3	253.79
	sucrose	2	4	8	3	241.8
NaCl concentration	sucrose	2	4	7	1	276.85
	sucrose	2	4	7	5	218.48

Table 2. Heavy metal tolerance by *Pseudoalteromonas* sp. MER144.



A: 10 ppm; B: 50 ppm; C: 100 ppm; D: 500 ppm; E: 1000 ppm; F: 2500 ppm; G: 5000 ppm; H: 7500 ppm; I: 10000 ppm.